Mechanisms of Steroid Oxidation by Microorganisms. XII. Metabolism of Hexahydroindanpropionic Acid Derivatives*

S. S. Lee† and Charles J. Sih

ABSTRACT: Three deoxo analogs of the steroid degradative intermediate, 7aβ-methyl-1,5-dioxo-3aα-hexahydro-4-indanpropionic acid (VII), have been synthesized and exposed to microorganisms. 7aβ-Methyl-5-oxo-3aα-hexahydro-4-indanpropionic acid (XII) was found to be transformed into 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indancarboxylic acid (XVIII) and 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indanpropionic acid (XIX); 7aβ-methyl-1β-hydroxy-3aα-hexahydro-4-indanpropionic acid (XIII) was found to be converted into 7aβ-methyl-1-oxo-3aα-hexahydro-4-indancarboxylic acid (XXI) and 7aβ-methyl-1-oxo-3aα-hexahydro-4-indanpropionic acid (XXII). On the other hand 7aβ-methyl-3aα-hexahydro-4-indanpropionic acid (XIV) was found to be inert to attack. The results suggest that the initial site of degradative attack by microorganisms involves a removal of two carbon atoms from the propionic acid side chain of VII, corresponding to carbon atoms 5 and 6 of the steroid skeleton.

Numerous microorganisms are capable of oxidizing the cyclopentanoperhydrophenanthrene nucleus to carbon dioxide and water (Turfitt, 1944; Schatz *et al.*, 1949). Previous studies (Dodson and Muir, 1961; Sih *et al.*, 1966; Gibson *et al.*, 1966; Sih and Wang, 1963; Wang and Sih, 1963) have shown that one degradative pathway of androst-4-ene-3,17-dione by microorganisms may be envisaged as follows: androst-4-ene-3,17-dione (I) → androsta-1,4-diene-3,17-dione (II) or 9α-hydroxyandrost-4-ene-3,17-dione (III) → 3-hydroxy-9,10-secoandrosta-1,3,5-(10)-triene-9,17-dione (V) → 4(5),9(10)-diseco-3-hydroxyandrosta-1(10),2-diene-5,9,-17-trion-4-oic acid (VI) → 7aβ-methyl-1,5-dioxo-3aα-hexahydro-4-indanpropionic acid (VII) + α-keto-Δ^3-hexenoic acid.

Our continued interest in defining the intermediates and reaction sequence, involved in the complete oxidation of the steroid nucleus, prompted us to investigate the further metabolism of the hexahydroindanpropionic acid VII. This paper provides evidence to indicate that the most probable first site of attack by microorganisms involves the removal of two carbon atoms from the propionic acid side chain in VII, corresponding to carbons 5 and 6 of the steroid skeleton.

Results

Nocardia restrictus, *Pseudomonas testosteroni*, *Mycobacterium rhodochrous*, and *Nocardia corallina* are organisms, capable of transforming androst-4-ene-3,17-dione (I) into 7aβ-methyl-1,5-dioxo-3aα-hexahydro-4-indanpropionic acid (VII) (Scheme I) (Sih and Wang, 1963). Furthermore, these organisms are capable of utilizing VII as a sole carbon source and rapidly metabolizing it to CO_2 and H_2O, without the apparent accumulation of degradative intermediates. However, when VII was exposed to *N. corallina* in high concentrations (3 mg/ml), two products were formed. These products were identified (by having identical mixture melting points and infrared spectra with authentic samples) to be 7aβ-methyl-5-oxo-1β-hydroxy-3aα-hexahydro-4-indanpropionic acid (VIII) and 7aβ-methyl-1-oxo-5α-hydroxy-3aα-hexahydro-4-indanpropionic acid δ-lactone (IX). This experiment showed that this microorganism possessed enzymes capable of catalyzing the interconversion of hydroxyl and keto functions at positions 1 and 5 in the hexahydroindane molecule.

When VIII was exposed to *P. testosteroni*, a product, mp 166–167°, was produced. It was assigned the structure, 7aβ-methyl-1β,5α-dihydroxy-3aα-hexahydro-4-indanpropionic acid δ-lactone (X) on the basis of its carbon–hydrogen analysis was in good agreement with $C_{13}H_{20}O_3$; its infrared spectrum showed bands at 2.94 (hydroxyl), 5.85 (carbonyl), and 8.03 μ (CO stretching). Its nuclear magnetic resonance (nmr) spectrum showed bands at τ 9.2 (3 H, C_{7a}-methyl), 6.34 (1 H, triplet, J = 4 cycles/sec, proton at C-1), and most significantly the peak at 5.43 (1 H, quadruplet J = 2 cycles/sec) suggesting that the proton on the carbon-bearing oxygen (C-5) possesses the equatorial configuration. It has previously been reported that the axial proton has much larger coupling constants and its chemical shift appeared at higher field (τ 6.10). The structure of X, was further confirmed by reduction

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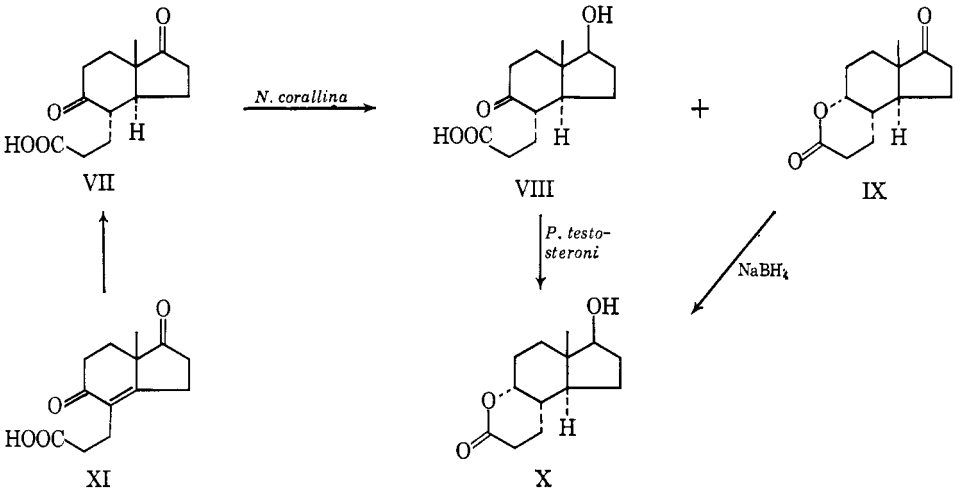
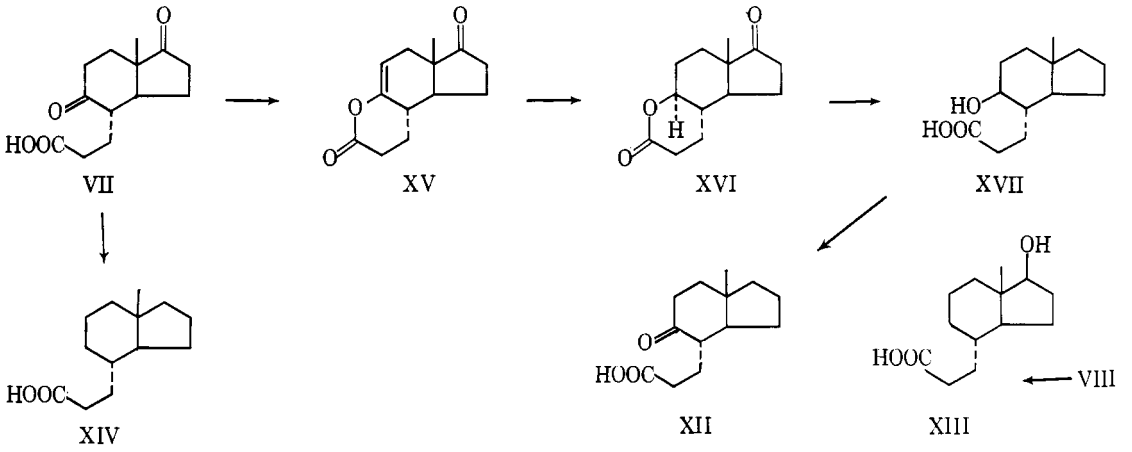
SCHEME I



SCHEME II

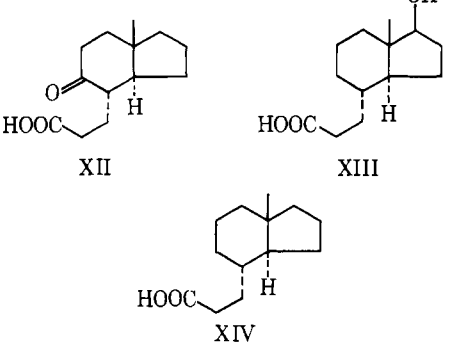


of IX with $NaBH_4$ to yield a compound, identical with X, with respect to mixture melting point and infrared spectrum. Although the chemical structures of these metabolites have been established, both IX and X were slowly metabolized by all the microorganisms listed above, indicating that they are probably not key intermediates in the degradation of VII.

Since all these microorganisms possess highly active steroid ring A dehydrogenases (Δ^1-3-keto, Δ^4-5α, and Δ^4-5β), it is possible that the first step in the metabolism of VII may involve an introduction of a double bond into the cyclohexanone portion of the molecule. Based on this rationale, 7aβ-methyl-1,5-dioxo-$\Delta^{4(3a)}$-tetrahydro-4-indanpropionic acid (XI) was exposed to *N. corallina*, but only VII and IX were obtained; similar results were obtained using other microorganisms.

As the conventional approach of exposing VII to microorganisms gave no new knowledge relative to its further metabolism, it appeared that an alterna tive approach to this problem is needed to surmoun this obstacle. Three functional groupings are present in the molecule (VII). Assuming that the first and most probable site of microbial attack involves one of these functional groups, one could envisage the following reactions to occur: oxygenation of the Baeyer–Villiger type on either the six-membered or five-membered ring carbonyl group to yield their respective lactones; such type of oxygenations have ample biochemical precedence (Fried *et al.*, 1953; Prairie and Talalay, 1963; Conrad *et al.*, 1965). Alternatively, the first site of attack may involve the β oxidation of the propionic acid side chain, *via* a manner analogous to the conventional fatty acid oxidation mechanism. If this line of reasoning should prove to be correct, one would expect that analogs of VII, lacking one or both carbonyl groups, when exposed to these organisms, should result in the accumulation of partially oxidized degradative intermediates.

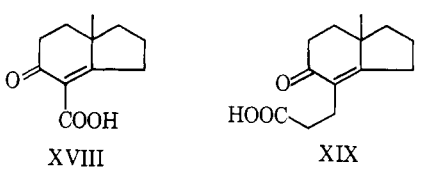
To test the validity of these assumptions, 7aβ-methyl-5-oxo-3aα-hexahydro-4-indanpropionic acid (XII), 7aβ-methyl-1β-hydroxy-3aα-hexahydro-4-indanpropionic acid (XIII), and 7aβ-methyl-3aα-hexahydro-4-indanpropionic acid (XIV) were synthesized *via* the following procedures.



Treatment of VII (Scheme II) with acetic anhydride and sodium acetate afforded the enol lactone XV; hydrogenation of XV gave the β-lactone XVI, which was converted into XVII *via* Wolf–Kishner reduction; oxidation of XVII with chromic trioxide in acetic acid afforded XII. The over-all yield of XII from VII was approximately 37%.

The starting material VIII was used for the preparation of XIII. Wolf–Kishner reduction of VIII (10 g) yielded 8.3 g of XIII, mp 91.5–93°; whose infrared spectrum exhibited bands at 3.00 (hydroxyl) and 5.93 μ (carboxyl carbonyl); its carbon–hydrogen analysis afforded values consistent for $C_{13}H_{22}O_3$. Similarly, VII was subjected to Wolf–Kishner reduction to give XIV, mp 49–50°; both the infrared and nmr spectra were in good agreement with the assigned structure.

When XII was incubated with *N. restrictus*, *N. corallina*, *N. erythropolis*, *N. opaca*, and *M. rhodochrous*, two ultraviolet-absorbing acids were accumulated. A large-scale fermentation of XII was carried out using *N. restrictus* since this microorganism appeared to give the best yields of products. The chemical structures of these two metabolites were assigned 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indancarboxylic acid (XVIII) and 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indanpropionic acid (XIX) on the basis of the following data.



Attempts to obtain an analytical sample of XVIII for carbon–hydrogen analysis by recrystallization were not successful, owing to the instability of the compound. Therefore, the crude crystalline product (mp 76–80°) was used directly for spectroscopic analysis and the preparation of derivatives. A mass spectrum of XVIII is shown in Figure 1. The most intensive peak in the parent region of the spectrum is at *m/e* 150. An isotopic analysis of the (P + 1) and (P + 2) values on this ion indicates it to be $C_{10}H_{14}O$. An intense peak (*m/e* 44) is also observed, indicating that the molecular weight of XVIII is probably 194 and that it lost CO_2 in the heated inlet of the apparatus. The fragmentation pattern (Budzikiewicz *et al.*, 1964) is also consistent with the proposed structure. The infrared spectrum of XVIII showed peaks at 3.63, 5.77, 6.15, and 6.25 μ; its ultraviolet spectrum in ethanol showed an absorption peak at 248 mμ (ε 11,500) and its nmr spectrum (Figure 2) in deuterated chloroform exhibited bands at τ 8.74 singlet (3 H, C-7a-methyl), 7.87–8.20 multiplet (6 H, methylenes), 7.27 multiplet (2 H, CH_2 adjacent to carbonyl), and 6.67 triplet (2 H, allylic protons at C-3). To further substantiate the proposed structure, XVIII was converted into its methyl ester (XX) by reaction with diazomethane. Unfortunately, it was found that XX was even more unstable than XVIII. Nonetheless, its infrared spectrum in Nujol showed peaks at 5.78 (carboxyl carbonyl), 6.00, and 6.10 μ (conjugated carbonyl). The nmr spectrum of XX showed peaks at τ 8.80 singlet (3 H, C-7a-methyl), 7.97–8.32 multiplet (6 H, methylenes), 7.67 multiplet (2 H, CH_2 adjacent to carbonyl), 7.30 broad (2 H, allylic protons at C-3), and 6.28 singlet (3 H, methoxy of methyl ester). All these data are in accord with the assigned structure XVIII.

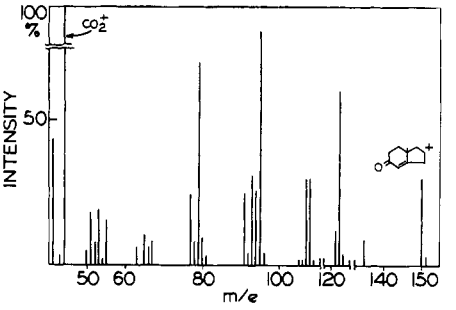


FIGURE 1: The mass spectrum of 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indancarboxylic acid (XVIII).

The chemical structure of the second metabolic product (XIX) was found to be identical with an authentic specimen of 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indanpropionic acid (XIX) (derived from fermentation of androstan-3-one with *N. restrictus*, Y. Abul-Hajj and C. J. Sih, unpublished data) with respect to melting point, mixture melting point, and infrared spectrum.

When the hexahydroindan derivative XIII was exposed to *N. restrictus*, two products were accumulated in the fermentation medium. The first product, mp 152–153°, was assigned the structural formula, 7aβ-methyl-1-oxo-3aα-hexahydro-4-indancarboxylic acid (XXI) on the basis of the following physical

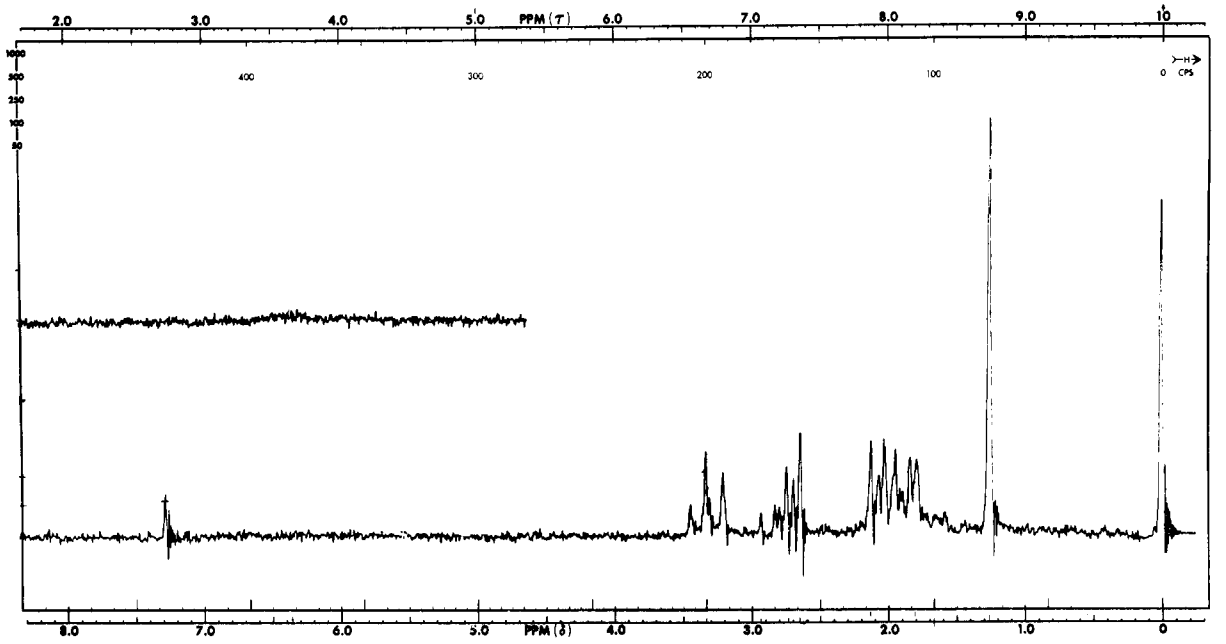
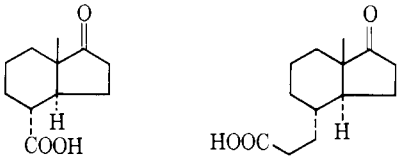


FIGURE 2: The nmr spectrum of 7aβ-methyl-5-oxo-$\Delta^{4(3a)}$-tetrahydro-4-indancarboxylic acid (XVIII).

data. Carbon–hydrogen analysis afforded values in good agreement with the empirical formula, $C_{11}H_{16}O_3$. The mass sepctrum of XXI (Figure 3) showed a parent ion peak at *m/e* 196 with (P + 1) and (P + 2) peaks compatible with the molecular formula. The nmr spectrum (Figure 4) in deuterated chloroform showed peaks at τ 9.08 singlet (3 H, tertiary CH_3), −1.00 singlet (1 H, COOH), and 12 protons buried between 7.2 and 8.7. The infrared spectrum in Nujol showed peaks at 5.81 and 3.05 μ. Treatment of XXI with diazomethane resulted in the formation of its methyl ester (XXIII), whose properties were all consistent with the assigned structures.

The identity of the other fermentation product (XXII) was established by direct comparison with an authentic sample of 7aβ-methyl-1-oxo-3aα-hexahydro-4-indanpropionic acid (XXII), obtained by chromic acid oxidation of XIII; their infrared spectrum, melting point, mixture melting point, and chromatographic behavior were found to be identical.



XXI XXII

When the hexahydroindane propionic acid XIV was exposed to all the microorganisms cited previously, it was found that none of these organisms metabolized XIV, even after 120 hr of incubation.

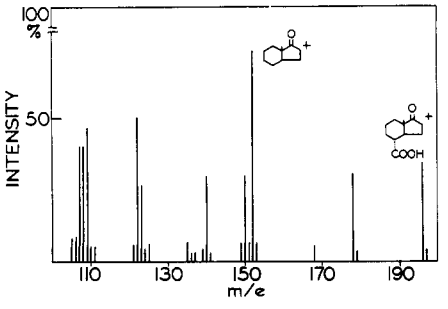


FIGURE 3: The mass spectrum of 7aβ-methyl-1-oxo-3aα-hexahydro-4-indancarboxylic acid (XXI).

Discussion

Considerable efforts have been expended in varying the fermentation conditions to detect key degradative intermediates of VII. Unfortunately, this approach had only led to the isolation of metabolic products as a result of side metabolic reactions involving oxidation and reduction of the carbonyl functions at C-1 and C-5, reduction and introduction of double bond at C-4(3a) position, and the lactonization of the 5-hydroxyl function with the propionic acid side chain.

Schubert *et al.* (1961) reported that 7aβ-methyl-5-oxo-1β-acetyl-3aα-hexahydro-4-indanpropionic acid (XXIII) is a degradative intermediate of progesterone. In subsequent papers, Schubert *et al.* (1964, 1965) reported the further transformation of XXIII with *Megabacterium smegmatis* into 7aβ-methyl-5-oxo-1β-acetyl-3aα-hexahydro-4-indanpropanol and 7aβ-methyl-1,5-dioxo-3aα-hexahydro-4-indanpropanol, accompa-

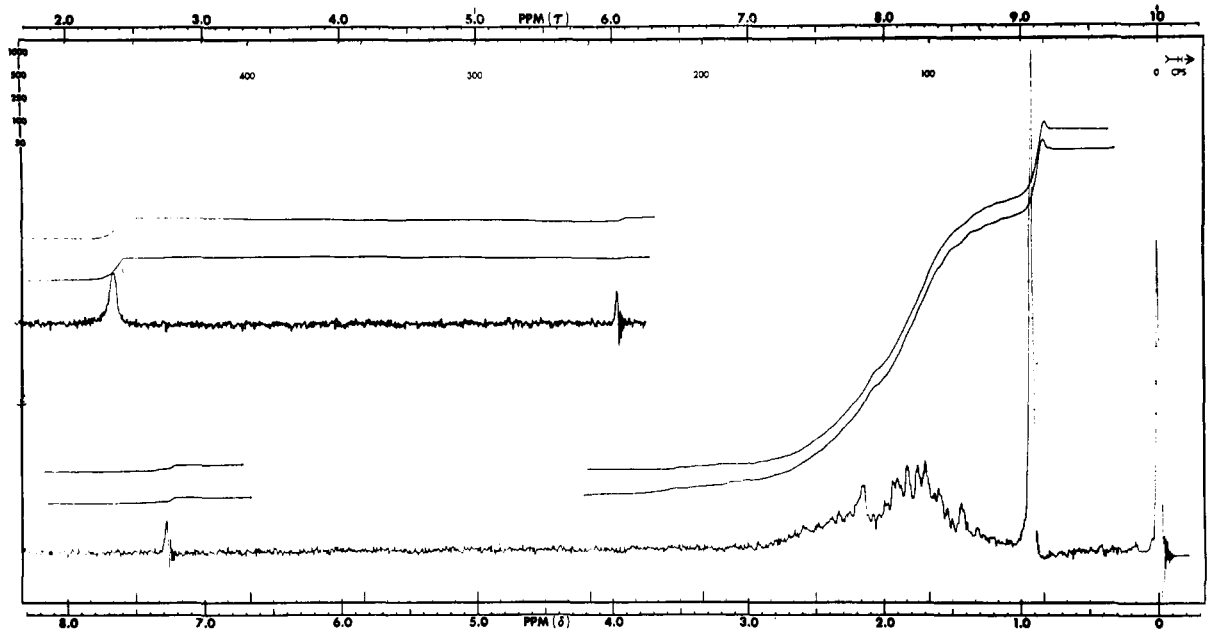


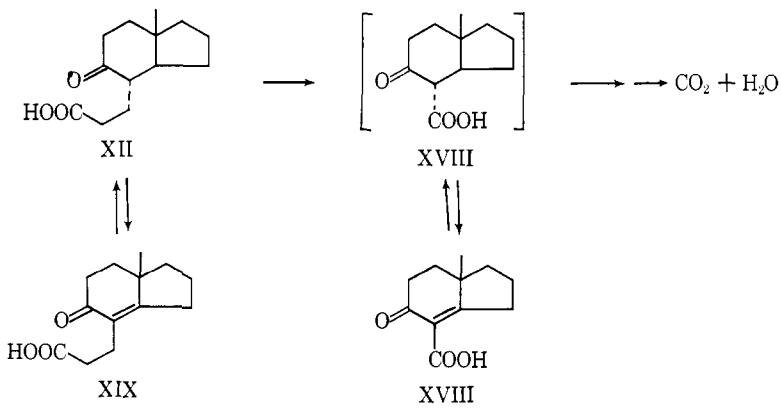
FIGURE: 4 The nmr spectrum of 7aβ-methyl-1-oxo-3aα-hexahydro-4-indancarboxylic acid (XXI).

nied by several other compounds where the two carbonyl functions in XXIII were reduced to their respective epimeric hydroxy derivatives. These results are consistent with our observations, in that microorganisms are capable of catalyzing the formation of numerous secondary metabolic products. Because of the transient nature of degradative intermediates it appears that the first degradative step of VII may well be the rate-limiting reaction.

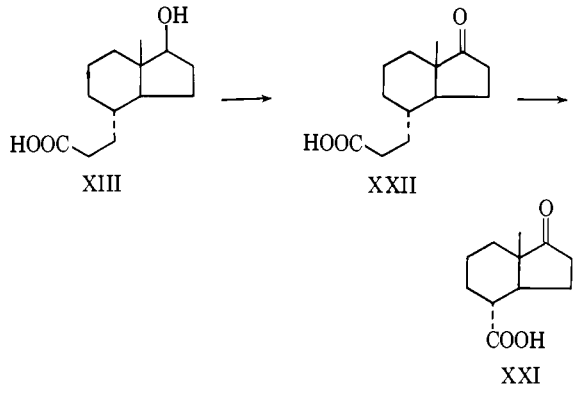
When XII (Scheme III) was exposed to *N. restrictus*, two products (XVIII and XIX) accumulated which suggested that the keto function at C-1 was required for the complete oxidation of the molecule. When XIX was reincubated with this organism, it was found that XIX was first converted back into XII and then to XVIII. Although inconclusive, this observation may be interpreted to mean that the dehydrogenation reaction is probably a side metabolic reaction rather than a step in the degradative sequence. This interpretation is further supported by the fact that when XI was exposed to the same organism, it was also first hydrogenated to VII, which was then further metabolized. Thus, the compound XVIII probably constitutes a secondary metabolite.

Exposure of XIII to *N. restrictus* resulted in an accumulation of XXI and XXII. However, XXI could not be further metabolized by the organism when reincubated. This observation suggests that the 5-oxo function may be required for further metabolism, such as cleavage of the six-membered ring. It is apparent that only one of the carbonyl functions at either C-1 or C-5 is required for the degradation of the propionic acid side chain. On the other hand, the removal of both of the carbonyl groups on the hexa-

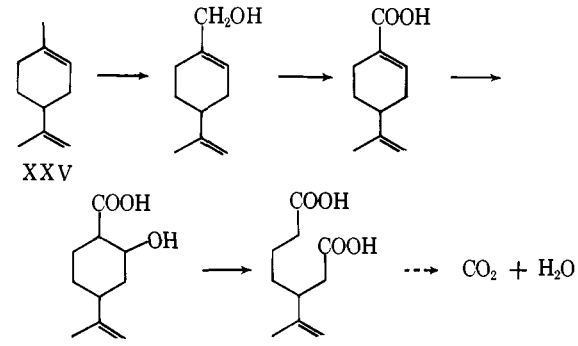
SCHEME III



hydroindanpropionic acid renders the molecule inert to attack, as XIV was not metabolized by all the microorganisms tested.



A recent report on the mode of degradation of limonene (XXV) (Ballal *et al.*, 1966) by a soil pseudomonad, has been proposed to proceed as follows.

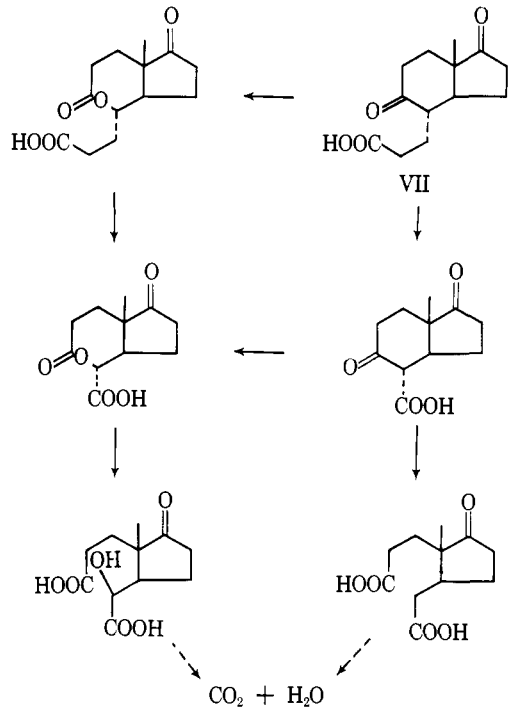


From the experimental data cited herein and biochemical analogy, the initial degradative sequence of VII (Scheme IV) may occur *via* a removal of two carbons from the propionic acid side chain in a manner analogous to the fatty acid oxidation mechanism, followed by the cleavage of the six-membered ring to yield a dibasic acid. Alternatively, the six-membered ring of VII may be opened by the Baeyer–Villiger-type oxidation (Laskin *et al.*, 1964) either before or after the attack on the propionic acid side chain to yield the corresponding lactones. These reactions are shown in Scheme IV.

Experimental Section

Materials. N. restrictus (ATCC 14887), *P. testosteroni* (ATCC 11996), *N. corallina*, and *M. rhodochrous* were kindly supplied by Dr. R. E. Kallio, University of Illinois, Urbana, Ill. The cultures were maintained on nutrient agar slants, supplemented with 1% yeast extract and 1% glucose. All solvents and inorganic chemicals were of reagent grade. Petroleum ether refers to the fraction with a boiling point of 40–70°. Silicic acid (Mallinckrodt 2847) was used for column chromatography and silica gel HF (Merck A. G. 7741) was used for thin layer chromatography (tlc).

SCHEME IV



Methods. Melting points, determined on a Thomas-Hoover melting point apparatus, are corrected. Ultraviolet absorption spectra were determined on a Cary Model 11 MS recording spectrophotometer and 95% ethanol was used as solvent. Infrared spectra were recorded on a Beckman IR 5A double-beam infrared recording spectrophotometer. Microanalyses were carried out by Mr. J. Alicino of Metuchen, N. J. Nmr spectra were determined on a Varian Associates recording spectrometer (A60A) at 60 Mcycles either in carbon tetrachloride or deuterated chloroform with tetramethylsilane as an internal standard. Chemical shifts are reported in τ values (parts per million) (Tiers, 1958). Mass spectra were taken by Morgan Shaffer Corp., Montreal 26, Quebec, Canada, on a Atlas CH 4 mass spectrometer, operating at an ionization of 70 v and employing a heated glass inlet system at 250°. Unless otherwise stated, the paper chromatographic system used throughout this work consisted of toluene–propylene glycol (Zaffaroni *et al.*, 1950). Values of $[\alpha]_D$ have been approximated to the nearest degree. The growth conditions for the microorganisms have been described previously (Sih *et al.*, 1966).

Transformation of 7aβ-Methyl-1,5-dioxo-3aα-hexahydro-4-indanpropionic Acid (VII) into 7aβ-Methyl-5-oxo-1β-hydroxy-3aα-hexahydro-4-indanpropionic Acid (VIII) and 7aβ-Methyl-1-oxo-5α-hydroxy-3aα-hexahydro-4-indanpropionic Acid δ-Lactone (IX). *N. corallina* was grown in 2.4 l. of Difco nutrient broth (six 2-l. erlenmeyer flasks), containing 0.1 M potassium phosphate buffer (pH 7.0). After 43 hr, 7.5 g of VII in

75 ml of 0.1 M KH_2PO_4 buffer (pH 7.0) was distributed equally among the flasks. After an additional 40 hr, the fermentation was terminated by the addition of dilute hydrochloric acid, and the cell debris was removed by filtration. The filtrate was extracted three times with ethyl acetate. The combined ethyl acetate extract (1.5 l.) was washed with water, dried over sodium sulfate, and evaporated to dryness to give 6.73 g of an oily residue. The residue was dissolved in 1 l. of a benzene–ethyl ether (1:1) mixture and was extracted three times with 6% sodium bicarbonate. The bicarbonate layer (800 ml) was acidified with 10% hydrochloric acid and extracted with chloroform. The combined chloroform solution was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue (3.8 g) was chromatographed over a silicic acid–Celite (95:5) column (3.5 × 40 cm). Elution of the column with a mixture of chloroform–methanol–acetic acid (99:1:0.1) afforded 105 mg of VIII, mp 153–155°; its infrared spectrum and melting point were identical with an authentic specimen.

The benzene–ether layer was washed with water, dried over sodium sulfate, and was evaporated to dryness to give 0.63 g of residue. The residue was chromatographed over a small silicic acid column (1 × 5 cm). Elution of the column with chloroform afforded 180 mg of the lactone IX, mp 125–127°, identical with an authentic specimen with respect to infrared spectrum and mixture melting point.

7aβ-Methyl-1β,5α-dihydroxy-3aα-hexahydro-4-indanpropionic Acid δ-Lactone (X). *P. testosteroni* was grown for 40 hr in 2 l. (five 2-l. erlenmeyer flasks) of the following medium: KH_2PO_4, 0.1%; K_2HPO_4, 0.1%; NH_4NO_3, 0.1%; $MgSO_4$, 0.02%; $CaCl_2$, 0.002%; and $FeCl_3$, 0.005%; hydrindanpropionic acid (VIII) 0.3%, and distilled water. The pH of the medium was adjusted to 7.0. After 12 hr of growth, the culture broth was acidified with 5 N sulfuric acid to pH 2.0 and the cell debris was removed by filtration. The filtrate was extracted three times with chloroform. The combined chloroform extract (1000 ml) was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue (1.7 g) was dissolved in 300 ml of a benzene–ether (1:1) mixture and was extracted three times with 100-ml portions of 6% sodium bicarbonate. The benzene–ether layer was dried over sodium sulfate and was evaporated to dryness to yield a crystalline residue. Recrystallization from acetone–petroleum ether gave the lactone X, mp 166–167°, identical with a sample (infrared and mixture melting point) prepared by $NaBH_4$ reduction of IX.

Borohydride Reduction of 7aβ-Methyl-1-oxo-5α-hydroxy-3aα-hexahydro-4-indanpropionic Acid δ-Lactone (IX). To 20 mg of IX dissolved in 1 ml of methanol was added 11 mg of $NaBH_4$ in 1 ml of methanol. The mixture was allowed to stand at room temperature for 2 hr. It was then acidified with 0.3 ml of 5 N H_2SO_4 and extracted with ethyl acetate. The ethyl acetate layer was dried over sodium sulfate and concentrated down to dryness to yield 15 mg of an oil. The oil was chromatographed on a silica gel HF plate (20 × 20 cm) and was developed with chloroform–acetone (80:20). The band was cut out and eluted with acetone to yield 4 mg of X, mp 166–167°, λ_{max}^{Nujol} 2.94 μ and 5.85 μ.

Anal. Calcd for $C_{13}H_{20}O_3$ (224.29): C, 69.61; H, 8.99. Found: C, 69.08; H, 8.96.

Metabolism of 7aβ-Methyl-1,5-dioxo-$\Delta^{4(3a)}$-tetrahydro-4-indanpropionic Acid (XI) by N. corallina. *N. corallina* was grown in 2 l. of Difco nutrient broth potassium phosphate buffer (0.1 M, pH 7.0) on a rotary shaker. After 40 hr, 6 g of XI was distributed equally to the flasks and the fermentation was continued for 40 hr. The reaction was terminated by acidification with hydrochloric acid and was extracted with ethyl acetate three times. The combined ethyl acetate solution (2000 ml) was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue (3.0 g) was dissolved in 1 l. of a benzene–ether (1:1) mixture and extracted with 6% sodium bicarbonate. The benzene–ether layer was washed with water, dried over sodium sulfate, and evaporated to dryness. The oily residue (0.75 g) was applied over a small silicic acid column (1 × 5 cm) and the column was eluted with chloroform to give 180 mg of IX, mp 124–127°. By working-up the bicarbonate fraction *via* the usual procedure, 138 mg of VII, mp 111–112°, was obtained.

3aβ-Methyl-1-oxo-5-hydroxy-$\Delta^{5(6)}$-3aα-tetrahydro-4-indanpropionic Acid δ-Lactone (XV). A mixture, containing 12 g of the acid VII, 7 g of anhydrous sodium acetate, and 300 ml of acetic anhydride were heated on an oil bath (85–90°) with stirring under an atmosphere of nitrogen. After evaporation of the solvent under reduced pressure, the residue was extracted three times with 1000 ml of ethyl ether. The ethereal solution was washed with 300 ml of 6% sodium bicarbonate, followed by 100 ml of water, and dried over sodium sulfate. The entire residue after removal of the solvent was used for hydrogenation without further purification.

7aβ-Methyl-1-oxo-5β-hydroxy-3aα-hexahydro-4-indanpropionic Acid δ-Lactone (XVI). The residue of the crude enolactone XV, dissolved in 350 ml of 95% ethanol, was mixed with 3.0 g of 10% palladium on carbon and stirred under hydrogen at atmospheric pressure at 25°. When the consumption of hydrogen gas stopped, the suspension was filtered and the filtrate was evaporated to dryness. Without further purification, this residue was used for the subsequent step.

7aβ-Methyl-5β-hydroxy-3aα-hexahydro-4-indanpropionic Acid (XVII). A mixture of 10 g of the crude XVI, 10 g of potassium hydroxide, 12 ml of 90% hydrazine hydrate, and 80 ml of diethylene glycol was refluxed in an oil bath (135–140°) for 12 hr. After removing the condenser, the temperature was increased to 195–200° and kept for 2 hr. The reaction mixture was cooled, diluted to 400 ml with water, and extracted three times with 500 ml of chloroform. The aqueous layer was acidified to pH 2.0 with dilute hydrochloric acid and was further diluted with water to 1 l. The acidified aqueous layer was extracted three times

with 600 ml of ethyl acetate, washed with water, and dried over sodium sulfate. The removal of the solvent under reduced pressure gave 11.2 g of an oily residue (XVII). Without further purification, the crude XVII was used directly for the next step.

7aβ-Methyl-5-oxo-3aα-hexahydro-4-indanpropionic Acid (XII). The crude compound XVII (11.2 g), dissolved in 100 ml of 95% acetic acid, was mixed together with 8.0 g of chromium trioxide in 400 ml of 95% acetic acid, and the mixture was left standing for 1 hr at 25°. Excess chromium trioxide was destroyed with 30 ml of ethanol. The volume of the reaction mixture was reduced to 100 ml by evaporation under reduced pressure. It was then diluted to 1 l. with water. The mixture was extracted three times with 800 ml of ethyl acetate, dried over sodium sulfate, and evaporated to dryness to yield an oily residue. This oily residue was applied over a silicic acid–Celite (95:5) column (5.8 × 60 cm) and was eluted with chloroform–methanol–acetic acid (98:1:0.1). Fractions containing XII were collected. After evaporation of the solvent, 4.5 g of crystalline single spot on thin layer plate, developed with chloroform–acetone–acetic acid (80:20:0.5) solid was obtained. Recrystallization from ethyl ether–petroleum ether gave XII, mp 119–120°; $[\alpha]_D^{26}$ +8° (*c* 1.0, $CHCl_3$); λ_{max}^{Nujol} 3.25, 5.78, and 5.95 μ.

Anal. Calcd for $C_{13}H_{20}O_3$ (224.29): C, 69.611; H, 8.99. Found: C, 69.92; H, 9.59.

7aβ-Methyl-1β-hydroxy-3aα-hexahydro-4-indanpropionic Acid (XIII). A mixture of 10 g of VIII, 8 g of sodium hydroxide, 10 ml of 85% hydrazine hydrate, and 90 ml of diethylene glycol was refluxed in an oil bath (135–140°) for 10 hr. After removing the condenser, the temperature was increased to 195–200° and kept for 2.5 hr. After cooling, the reaction mixture was extracted by the same procedure described earlier. An oily residue (9.2 g) was obtained. Two crystallizations from ethyl ether–petroleum ether gave an analytical sample, mp 91.5–93°; $[\alpha]_D^{25}$ −27.8° (*c* 2.1, $CHCl_3$); λ_{max}^{Nujol} 3.00, 3.67, and 5.93 μ.

Anal. Calcd for $C_{13}H_{22}O_3$ (226.31): C, 68.91; H, 9.80. Found: C, 68.81; H, 9.97.

7aβ-Methyl-3aα-hexahydro-4-indanpropionic Acid (XIV). A mixture of 2 g of VII, 3 g of potassium hydroxide, and 20 ml of diethylene glycol was refluxed on an oil bath (135–140°) for 20 hr. After removing the condenser, the temperature was increased to 195–200° and kept for 2 hr. The reaction mixture was worked up in the same way as described previously. The oily residue was applied over a cellulose–powder column (58 × 4.5 cm) and the column was eluted with benzene–cyclohexane (30:70), saturated with propylene glycol; 16-ml fractions were collected. Fraction 15–30 were pooled together, and the removal of the solvent gave an oily residue. This oily residue was dissolved in 200 ml of 1 N potassium carbonate solution and was extracted with chloroform. The aqueous layer was acidified with dilute hydrochloric acid and extracted two times with 200 ml of ethyl acetate. The removal of the solvent afforded an oily residue (600 mg) which eventually crystallized, mp 42–45°. The noncrystallizable portion of this compound was applied over a small silicic acid column and the column was eluted with chloroform–acetic acid (100:0.1). Fractions containing XV were pooled and evaporated to dryness to give 520 mg of a crystalline compound, mp 49–50°, λ_{max}^{Nujol} 3.58–3.80 and 5.85 μ. The 5.85-μ peak was shifted to 6.38 μ by carboxylate formation with potassium hydroxide, $[\alpha]_D^{26}$ −17° (*c* 1.0, $CHCl_3$).

Anal. Calcd for $C_{13}H_{22}O_2$ (210.31): C, 74.24; H, 10.54. Found: C, 73.94; H, 10.27.

Formation of 7aβ-Methyl-5-oxo-3aα-hexahydro-4-indanpropionic Acid. N. restrictus (300 ml), grown in Difco nutrient broth for 24 hr, was transferred into 6 l. of Difco nutrient broth (15 2-l. erlenmeyer flasks). After 24 hr on a rotary shaker, 2.1 g of XII, dissolved in 45 ml of dimethylformamide was distributed equally among the flasks. After an additional 5 days, the fermentation broth was acidified with glacial acetic acid to pH 3.0 and the cell debris was removed by filtration. The filtrate was extracted with chloroform three times. The combined chloroform extract (5 l.) was washed with water, dried over sodium sulfate, and evaporated to dryness under reduced pressure without heating.

The oily residue (2.05 g) was applied over a silicic acid–Celite (95:5) column (4.5 × 60 cm) and the column was eluted with chloroform–methanol–acetic acid (99:1:0.1); 18-ml fractions were collected. Fractions 138–149 were pooled together and the removal of the solvent gave 240 mg of a violet residue. This residue was crystallized from ethyl ether–petroleum ether to yield XVIII, mp 76–80°; $\lambda_{max}^{alcohol}$ 248 mμ (ε 11,500); $\lambda_{max}^{CHCl_3}$ 3.63, 5.77, 6.15, 6.25, and 7.00 μ. Because of its instability, further purification through recrystallization was avoided. This compound was kept at low temperature and was used directly for spectrophotometric analyses and the preparation of derivatives.

Fractions 164–200 were pooled together and the removal of the solvent gave 150 mg of a strongly colored residue. This residue was again applied over a cellulose–powder column (3.5 × 57 cm) and the column was eluted with benzene, saturated with propylene glycol; 12-ml fractions were collected. Fractions 28–35 were pooled together. The volume of the benzene solution was reduced to 30 ml by evaporation, and was then washed three times with 100 ml of 6% sodium bicarbonate. The bicarbonate layer was acidified with dilute hydrochloric acid, extracted with 40 ml of chloroform, and dried over sodium sulfate. The removal of the solvent afforded 82 mg of crystalline residue. Recrystallization from ethyl ether–petroleum ether gave XIX, mp 153–155°; $[\alpha]_D^{26}$ +73° (*c* 1.0, $CHCl_3$); λ_{max}^{Nujol} 3.79, 3.93, 5.75, and 6.03 μ.

Anal. Calcd for $C_{13}H_{18}O_3$ (222.27): C, 70.24; H, 8.16. Found: C, 70.16; H, 7.97.

Methylation of XVIII. To 50 mg of XVIII in 20 ml of ethyl ether, an ethereal solution of diazomethane was added dropwise until the mixture remained a faint yellow color. After wrapping the flask with aluminum foil, the reaction mixture was left standing at room

temperature for 2 hr. The solvent was removed by aeration. The residue was applied over a small cellulose-powder column, and the column was eluted with cyclohexane, saturated with propylene glycol. Fractions containing XX were pooled together, washed with water, dried over sodium sulfate, and evaporated to dryness to yield 43 mg of XX as an oil. This oil was eventually crystallized in the cold. Further purification by recrystallizations were unsuccessful because of its instability. Its infrared spectrum in Nujol showed bands at 5.78, 6.00, 6.10, 8.27, and 8.79 μ.

Fermentation of 7aβ-Methyl-1β-hydroxy-3aα-hexahydro-4-indanpropionic Acid (XIII). *N. restrictus* (300 ml), grown in Difco nutrient broth for 24 hr, was transferred into 6 l. of Difco nutrient broth (15 2-l. erlenmeyer flasks). After 24 hr on a rotary shaker, 6.0 of XIII, dissolved in 45 ml of DMF, was distributed equally among the flasks. After an additional 16 hr, the fermentation broth was acidified with glacial acetic acid to a pH of 2.0. After removing the cell debris, the filtrate was extracted in the usual manner (same as in the fermentation of XII). An oily residue (6.7 g) was obtained which was applied over a cellulose-powder column (7.5 × 62 cm). The column was eluted with isooctane–benzene, and then benzene, saturated with propylene glycol. Fractions of 17 ml were collected and the elution profile is listed in Table I. After fraction 320, the column was eluted with methanol and the recovered residue was mainly XIII.

TABLE I: Elution Profile.

Fraction	Eluent (isooctane–benzene)	Wt (mg)	Compound
1–66	50:50	–	–
63–145	25:75	–	–
146–180	25:75	870	XXI
181–195	25:75	1180	XXI and XXII
196–320	0:100	1780	XXII

Fractions 146–180 were pooled together; the removal of the solvent gave an oily residue. The oily residue was dissolved in chloroform and was washed with 2% sodium carbonate. The carbonate layer was acidified with dilute hydrochloric acid and was extracted again with chloroform. The combined chloroform extract was washed with water and dried over sodium sulfate. The removal of the solvent gave 870 mg of XXI. Recrystallization from ethyl ether–petroleum ether gave an analytical sample of XXI, mp 109–110°; $[\alpha]_D^{25}$ +77.5° (*c* 2.67, $CHCl_3$); λ_{max}^{Nujol} 3.20, 5.78, and 5.83 μ.

Anal. Calcd for $C_{13}H_{20}O_3$ (224.29): C, 69.61; H, 8.99. Found: C, 69.66; H, 9.10.

Fractions 196–320 were pooled together and worked up in the same manner as XXI. Recrystallization from ethyl ether–petroleum ether gave an analytical sample (XXII) mp 152–153°; $[\alpha]_D^{25}$ +71° (*c* 2.69, $CHCl_3$); λ_{max}^{Nujol} 3.05, 5.79, and 5.82 μ.

Anal. Calcd for $C_{11}H_{16}O_3$ (196.24): C, 67.32; H, 8.22. Found: C, 67.33; H, 8.37.

Methylation of XXII. Reaction of XXII (100 mg) with diazomethane in ether for 2 hr afforded 105 mg of a residue, which was passed over a small silicic acid column. Elution of the column with chloroform afforded a noncrystallizable oil (mp 10°); infrared spectrum (liquid film): λ_{max} 5.76, 5.79, 8.62, and 8.93 μ.

References

Ballal, N. R., Bhattacharyya, P. K., and Rangachari, P. N. (1966), *Biochem. Biophys. Res. Commun. 23*, 473.

Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1964), Interpretation of Mass Spectra of Organic Compounds, San Francisco, Calif., Holden-Day, p 155.

Conrad, H. E., Lieb, K., and Gunsalus, I. C. (1965), *J. Biol. Chem. 240*, 4029.

Dodson, R. M., and Muir, R. D. (1961), *J. Am. Chem. Soc. 83*, 4627.

Fried, J., Thoma, R. W., and Klinsberg, A. (1953), *J. Am. Chem. Soc. 75*, 5764.

Gibson, D. T., and Wang, K C., Sih, C. J., and Whitlock, H. (1966), *J. Biol. Chem. 241*, 551.

Laskin, A., Grabowich, P., Meyers, C. D., and Fried, J. (1964), *J. Med. Chem. 7*, 406.

Prairie, R. L., and Talalay, P. (1963), *Biochemistry 2*, 203.

Rahim, M. A., and Sih, C. J. (1966), *J. Biol. Chem. 241*, 3615.

Sih, C. J., Lee, S. S., Tsong, Y. Y., and Wang, K. C. (1966), *J. Biol. Chem. 241*, 540.

Sih, C. J., and Wang, K. C. (1963), *J. Am. Chem. Soc. 85*, 2135.

Schatz, A., and Savard, K., and Pintner, I. J. (1949), *J. Bacteriol. 58*, 117.

Schubert, K., Bohme, K. H., and Horhold, C. (1961), *Z. Physiol. Chem. 325*, 260.

Schubert, K., Bohme, K. H., and Horhold, C. (1964), *Steroids 4*, 581.

Schubert, K., Bohme, K. H., and Horhold, C. (1965), *Biochim. Biophys. Acta 111*, 529.

Tiers, G. V. D. (1958), *J. Phys. Chem. 62*, 1151.

Turfitt, G. E. (1944), *J. Bacteriol. 47*, 487.

Wang, K. C., and Sih, C. J. (1963), *Biochemistry 2*, 1238.

Zaffaroni, A., Burton, R. B., and Keutman, E. H. (1950), *Science 111*, 6.